Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Toxicology and Applied Pharmacology 414 (2021) 115412

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/taap

Chloroquine may induce endothelial injury through lysosomal dysfunction and oxidative stress

PauloC. Gregório [a], Regiane S. da Cunha [a], Gilson Biagini [b,1], Bruna Bosquetti [a], Júlia Budag [a], Alberto Ortiz [d,e], Maria Dolores Sánchez-Niño [e,f], Fellype C. Barreto [c], Andréa E.M. Stinghen [a,*]

[a] Experimental Nephrology Laboratory, Basic Pathology Department, Universidade Federal do Paraná, Curitiba, PR, Brazil
[b] Department of Internal Medicine, Universidade Federal do Paraná, Curitiba, PR, Brazil
[c] Department of Internal Medicine, Division of Nephrology, Universidade Federal do Paraná, Curitiba, PR, Brazil
[d] Nephrology and Hypertension, Hospital Universitario Fundación Jiménez Díaz, Madrid, Spain
[e] IIS-Fundación Jimenez Diaz UAM, Madrid, Spain
[f] Department of Pharmacology, School of Medicine, Universidad Autonoma de Madrid, Madrid, Spain

ARTICLE INFO

Keywords:
COVID-19
Therapy
Fabry disease
Endothelial cells
Oxidative stress
Agalsidase-β

ABSTRACT

COVID-19 is a pandemic with no end in sight. There is only one approved antiviral agent but global stocks are deemed insufficient. Despite *in vitro* antiviral activity, clinical trials of chloroquine and hydroxychloroquine were disappointing, and they may even impair outcomes. Chloroquine causes zebroid deposits reminiscent of Fabry disease (α-galactosidase A deficiency) and endothelial cells are key targets of COVID-19. We have explored the effect of chloroquine on cultured endothelial cells and its modulation by recombinant α-galactosidase A (agalsidase). Following dose-response studies, 0.5 μg/mL chloroquine was added to cultured human endothelial cells. Neutral red and Lysotracker were used to assess lysosomes. Cytotoxicity was evaluated by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) - MTT assay and cell stress by assessing reactive oxygen species (ROS) and nitric oxide (NO). In endothelial cells, chloroquine induced dose-dependent cytotoxicity at *in vitro* test concentrations for COVID-19 therapy. At a sublethal concentration, chloroquine significantly induced the accumulation of acid organelles ($P < 0.05$), increased ROS levels, and decreased NO production ($P < 0.05$). These adverse effects of chloroquine on endothelial cell biology were decreased by agalsidase-β ($P < 0.05$). Chloroquine-induced endothelial cell cytotoxicity and stress is attenuated by agalsidase-β treatment. This suggests that endothelial cell injury may contribute to the failure of chloroquine as therapy for COVID-19 and may be at least in part related to causing dysfunction of the lysosomal enzyme α-galactosidase A.

1. Introduction

The current coronavirus disease-2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has already caused at least one million deaths worldwide (Coronavirus Disease, 2021). Currently, there is no end in sight to the pandemic. There is not yet a universally available vaccine and the disease had a rapid expansion in Europe, Asia and most of the American countries. Lethality is still high despite recent evidence that corticosteroids and the antiviral remdesivir may be of benefit (Wang et al., 2020a). In this regard, corticosteroids are a late intervention and it would be preferable to stop the virus prior to the development of severe viremia and an uncontrolled inflammatory response (Carriazo et al., 2020). A very active search for already available drugs that may be repurposed to decrease the severity of COVID-19 so ingoing. Thus, clinical trials are exploring the impact of drugs such as statins and SGLT2 inhibitors, among others (Fernandez-Fernandez et al., 2020; Rodrigues-Diez et al., 2020). However, the world attention focused on chloroquine and hydroxychloroquine, two cheap, widely available oral drugs used to treat malaria and some autoimmune conditions (Colson et al., 2020).

Chloroquine has *in vitro* activity against several virus, including SARS-CoV-2 (Wang et al., 2020b). These drugs increase the pH of

* Corresponding author at: Experimental Nephrology Laboratory, Basic Pathology Department, Universidade Federal do Paraná, Rua XV de novembro, 1299 – Centro, Curitiba – PR CEP: 80060-000, Brazil.
E-mail address: andreastinghen@ufpr.br (A.E.M. Stinghen).
[1] In memoriam.

https://doi.org/10.1016/j.taap.2021.115412
Received 26 October 2020; Received in revised form 23 December 2020; Accepted 11 January 2021
Available online 21 January 2021

endosomes that the virus uses for cell entry and may also interfere with the glycosylation of the cellular receptor of SARS-CoV-2 (White et al., 2020). Activities (EC50s) against the SARS-CoV-2 virus are in the low micromolar range which represents the upper end of the safely achievable free plasma concentration range (White et al., 2020). To avoid toxicity, dose regimens should aim to achieve whole blood concentrations below 10 μM (3.2 μg/mL), corresponding approximately to plasma concentrations <3 μM (1 μg/mL) (White et al., 2020). Early optimistic reports from uncontrolled trials and the wide availability of these well-known drugs transformed them into the standard of therapy in many countries as no other weapons were known. However, data from clinical trials have not confirmed the benefit of chloroquine or hydroxychloroquine neither in prophylaxis nor therapy. After high-risk or moderate-risk exposure to COVID-19, hydroxychloroquine did not prevent COVID-19 when used as postexposure prophylaxis within 4 days after exposure (Boulware et al., 2020). Moroever, hydroxychloroquine may even be deleterious. In the British Randomised Evaluation of COVID-19 therapy (RECOVERY), a trend toward increased mortality was observed in patients allocated to hydroxychloroquine *versus* usual care (rate ratio 1.09; 95% confidence interval [CI] 0.96 to 1.23; $P = 0.18$). Furthermore, significantly less patients on hydroxychloroquine were discharged from hospital alive within 28 days (60.3% *vs.* usual care 62.8%; rate ratio 0.92; 95% CI 0.85–0.99) and hydroxychloroquine patients not on invasive mechanical ventilation at baseline were more likely to reach the composite endpoint of invasive mechanical ventilation or death (29.8% *vs.* 26.5%; risk ratio 1.12; 95% CI 1.01–1.25) (Horby et al., 2021.). Understanding the disappointing clinical outcomes despite the promising *in vitro* antiviral effect may help to guide therapy for COVID-19. In this regard, the decrease in endosomal pH may cause dysfunction of lysosomal enzymes, increased lysosomal size and the development of zebroid bodies, similar to those found in Fabry disease (FD), is a classical finding in chloroquine-treated patients (Costa et al., 2013).

FD is a rare X-linked disease characterized by deficient activity of the α-galactosidase A (α-GAL) enzyme due to mutations in the GLA gene. This enzyme deficiency leads to lysosomal accumulation of glycosphingolipids possessing a terminal α-galactosyl moiety, including globotriaosylceramide (Gb3) and its deacylated metabolite, globotriaosylsphingosine (lyso-Gb3) (Desnick, 2015; Germain, 2010). Endothelial cell injury is a primary driver of FD complications. Notably, Gb3 accumulation may decrease the expression of endothelial nitric oxide synthase (eNOS) and the bioavailability of nitric oxide (NO) (Park et al., 2008; Kang et al., 2014), ultimately leading to impaired endothelial NO-mediated vasodilation, which could affect blood pressure (Park et al., 2008). Interestingly, FD is associated with an imbalance of redox homeostasis and increased oxidative stress, with consequent damage to cellular biomolecules, such as proteins, lipids and DNA (Schieber and Chandel, 2014; Ravarotto et al., 2018; Biancini et al., 2012; Chimenti et al., 2015). Gb3 may also up-regulate the expression of plasminogen activator inhibitor (PAI) and cell adhesion molecules such as vascular cell adhesion molecule-1 (sVCAM-1), P-selectin, and *E*-selectin (Shu et al., 2014; Shen et al., 2008). Enzyme replacement therapy (ERT) for FD consists of replacing the missing or hypofunctional enzyme by administering recombinant human α-GAL (agalsidase).

Endothelial cell injury and a prothrombotic state have emerged as key pathogenic features of COVID-19. Additionally, chloroquine toxicity (*i.e* cardiac arrhythmia) partially overlaps with FD symptoms. Thus, the present study hypothesized that chloroquine may cause endothelial cell injury through mechanisms involving interference with lysosomal function, which could be modulated by agalsidase.

2. Materials and methods

2.1. Reagents

Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), and penicillin/streptomycin were purchased from Gibco (Grand Island, USA). Fluoromount G and DAPI were obtained from Life Technologies (Carlsbad, USA). LysoTracker probe, 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) reactive oxygen species (ROS)-sensitive fluorescent dye, and the NO specific fluorescent probe DAF-FM Diacetate were commercially obtained (Thermo Scientific, Massachusetts, USA). [4, 5-dimethyl-thiazol-2-yl] -2, 5-diphenyltetrazolium bromide (MTT), neutral red solution (NR), and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, USA). Agalsidase-β and chloroquine were purchased from Genzyme (Boston, USA) and Cristália (São Paulo, Brazil), respectively. All other reagents were obtained from Sigma-Aldrich (St. Louis, USA) if not otherwise specified.

2.2. Endothelial cell culture and treatment conditions

The immortalized human endothelial cell line EA.hy926 (ATCC CRL 2922, Virginia, USA) was cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 10 mg/mL of penicillin/streptomycin and was maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Endothelial cells were exposed to chloroquine (Inagaki et al., 1993). A dose-response curve experiments for chloroquine (0.05, 0.1, 0.5, 1, 2, and 3 μg/mL) and agalsidase-β (5, 10, and 15 μg/mL) for 72 h were performed. Based on the results of these experiments, 0.5 μg/mL of chloroquine and 5 μg/mL of agalsidase-β were established as experimental conditions.

2.3. Cell viability assay

Cell viability was assessed using the MTT assay as described previously (Mosmann, 1983). Briefly, endothelial cells were plated into 96-well culture plates at a density of 2.5×10^3 cells per well. After 24 h of incubation, the medium was replaced, and the cells were treated with chloroquine and agalsidase-β for 72 h. This medium was then replaced with fresh medium (100 μL/well), and 10 μL of MTT (Sigma-Aldrich, Missouri, USA) solution (5 mg/mL in D-PBS) was added to each well. The plate was then further incubated for 4 h at 37 °C. Subsequently, the media was removed and replaced with DMSO to dissolve the crystals of reduced formazan, and the absorbance was then measured at 570 nm (Tecan, Männedorf, Switzerland). All analyses were performed in triplicate.

2.4. Acid organelle accumulation assay

Endothelial cells were plated into 96-well plates (2.5×10^3 cells/well) and treated with chloroquine and agalsidase-β for 72 h. Next, all medium was removed, and 100 μL NR solution (40 μg/mL) was added. Subsequently, the plate was incubated for 3 h at 37 °C and 5% CO_2. Finally, the NR-containing medium was removed, the cells were washed with 150 μL PBS per well, and 150 μL of developer solution (1% glacial acetic acid, 48% ethanol) was added. The absorbance was measured using a 540 nm filter spectrophotometer (Repetto et al., 2008). NR stains lysosomes as colour is pH-dependent. After the NR assay, the solution was removed from the wells and washed once with PBS (200 μL/well). Next, 100 μL of violet crystal solution (0.25 mg/mL) was added per well, and the plates were incubated for 20 min at room temperature (22 °C). Then, the wells were washed 2× with PBS and solubilized with 33% acetic acid. Finally, the absorbance was measured using a spectrophotometer at a wavelength of 570 nm. The result was obtained by normalizing the NR test to the violet crystal. Three experiments were performed in quintuplicate.

2.5. Fluorescence lysosome inclusions visualization assay

The LysoTracker fluorescent probe was used for visualization of acid organelle inclusions such as lysosomes since lysosome size is related to amount of inclusions. The cells were plated on coverslips and were

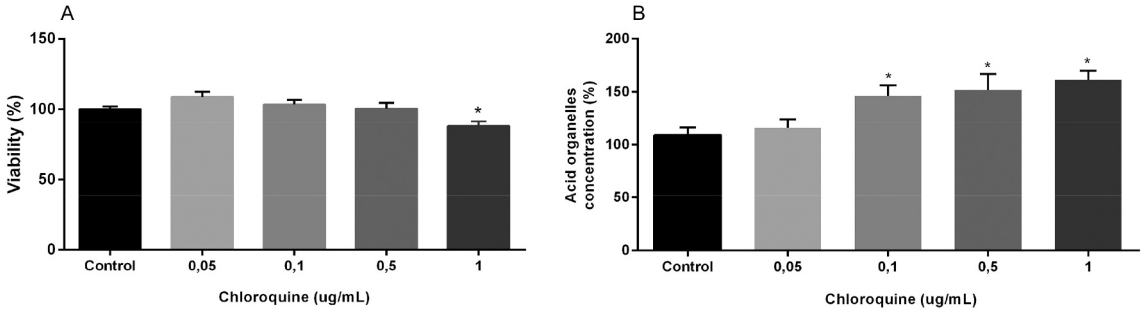

Fig. 1. Chloroquine decreases endothelial cell viability and induces acid organelles. Endothelial cells were incubated with various concentrations of chloroquine (0.05, 0.1, 0.5 and 1 μg/mL) or vehicle (DMEM) for 72 h. Cell viability and acid organelles were assessed using the MTT and neutral red methods, respectively. (A) Cell viability. * $P < 0.05$ 1 μg/mL chloroquine *vs.* control. (B) Acid organelles. * $P < 0.05$, 0.1, 0.5, and 1 μg/mL chloroquine *vs.* control. Results expressed as % of control (vehicle) and represent the mean ± SEM of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

treated with chloroquine and agalsidase-β as described above. The treatment medium was removed, and cells were washed twice with sterile PBS. Next, cells were incubated for 90 min at 37 °C with 50 nM of LysoTracker probe diluted in culture medium and subsequently washed twice with PBS, fixed with 2% paraformaldehyde for 20 min, and washed again with PBS. The coverslips were mounted on histological slides with Fluoromont-GTM and DAPI mounting medium, sealed with formaldehyde-free colour enamel, and observed under a Nikon A1RSiMP confocal microscope (NIKON, Tokyo, Japan). Three experiments were performed in duplicate.

2.6. ROS and NO production

DCFH-DA, a ROS-sensitive fluorescent dye, was used to measure ROS production (mostly peroxides), and NO was assessed using the NO-specific fluorescent probe DAF-FM Diacetate. Endothelial cells (2.5×10^3 cells/well) were seeded into a black 96-well plate. After 24 h of culture, cells were treated with chloroquine and agalsidase-β with or without *N*-acetyl-L-cysteine (NAC, 2 mM), an antioxidant, and N-nitro-L-arginine methyl ester (L-NAME, 100 μM), an inhibitor of nitric oxide synthase (eNOS) activity, for 72 h. Next, cells were washed with PBS at 37 °C and labelled with 1 μM DCFH-DA or 1 μM DAF-FM for 30 min at 37 °C, washed twice with PBS. Fluorescence was measured immediately using a spectrofluorometer to assess ROS (λEx 504 nm, λEm at 524 nm) and NO (λEx 495 nm, λEm at 515 nm). Data were expressed as the percentage increase in fluorescence intensity *versus* untreated cells (control).

2.7. Data analysis

Statistical analyses were performed using the statistical packages JMP (version 8.0; SAS Institute Inc., Cary, N.C., USA) and SigmaStat (version 3.5; Systat software Inc., Erkrath, Germany). Comparisons between groups were performed using a Student's *t*-test or an analysis of variance (ANOVA) for paired data and using Mann-Whitney and ANOVA on Rank's for unpaired data. Values were expressed as mean ± standard error of the mean (SEM). Three or five independent experiments were performed. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Chloroquine decreases endothelial cell viability

To select an appropriate chloroquine concentration, a dose-response curve experiment was performed to evaluate cell viability and the accumulation of acid organelles in endothelial cells at different chloroquine concentrations (0.05, 0.1, 0.5, and 1 μg/mL). A chloroquine concentration of 1 μg/mL significantly reduced the viability of endothelial cells ($P < 0.05$) (Fig. 1A). A chloroquine concentration of 0.5 μg/mL was the highest concentration that increased the accumulation of acid organelles ($P < 0.05$) without reducing cell viability, and this was the concentration used for all experiments (Fig. 1B).

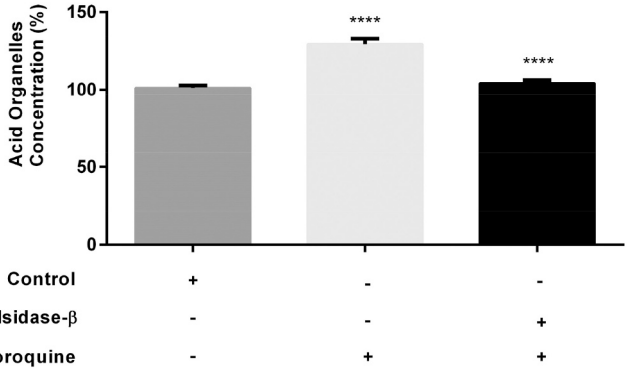

Fig. 2. Chloroquine promotes the accumulation of acid organelles in endothelial cells and this is prevented by agalsidase-β. Endothelial cells were incubated with chloroquine (0.5 μg/mL) and agalsidase-β (5 μg/mL) for 72 h. The dose of chloroquine was based on dose-response studies shown in Fig. 1. Acid organelle accumulation was assessed using the neutral red assay. Control (non-treated cells), agalsidase-β (5 μg/mL), and chloroquine (0.5 μg/mL) cells were studied. **** $P < 0.0001$; chloroquine *vs.* control and Agalsidase-β 5 μg/mL *vs.* chloroquine. Results are expressed as % of control (non-treated cells) and represent the mean ± SEM of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Agalsidase-β reduces the accumulation of acid organelles in cultured endothelial cells exposed to chloroquine

Once an endothelial cell culture system was established that was clinically relevant from the point of view of the chloroquine concentration used, a dose-response curve experiment was performed to verify the concentration of agalsidase-β required to reduce the accumulation of acid organelles in endothelial cells treated with chloroquine. There was a significant increase in acid organelles ($P < 0.0001$) in cells treated with chloroquine at 0.5 μg/mL compared to levels in control cells. Endothelial cells treated with agalsidase-β at 5 μg/mL and chloroquine at 0.5 μg/mL exhibited a significantly lower accumulation of acid organelles than observed after chloroquine alone ($P < 0.0001$) (Fig. 2). This 5 μg/mL concentration of agalsidase-β did not significantly alter cell viability (data not shown).

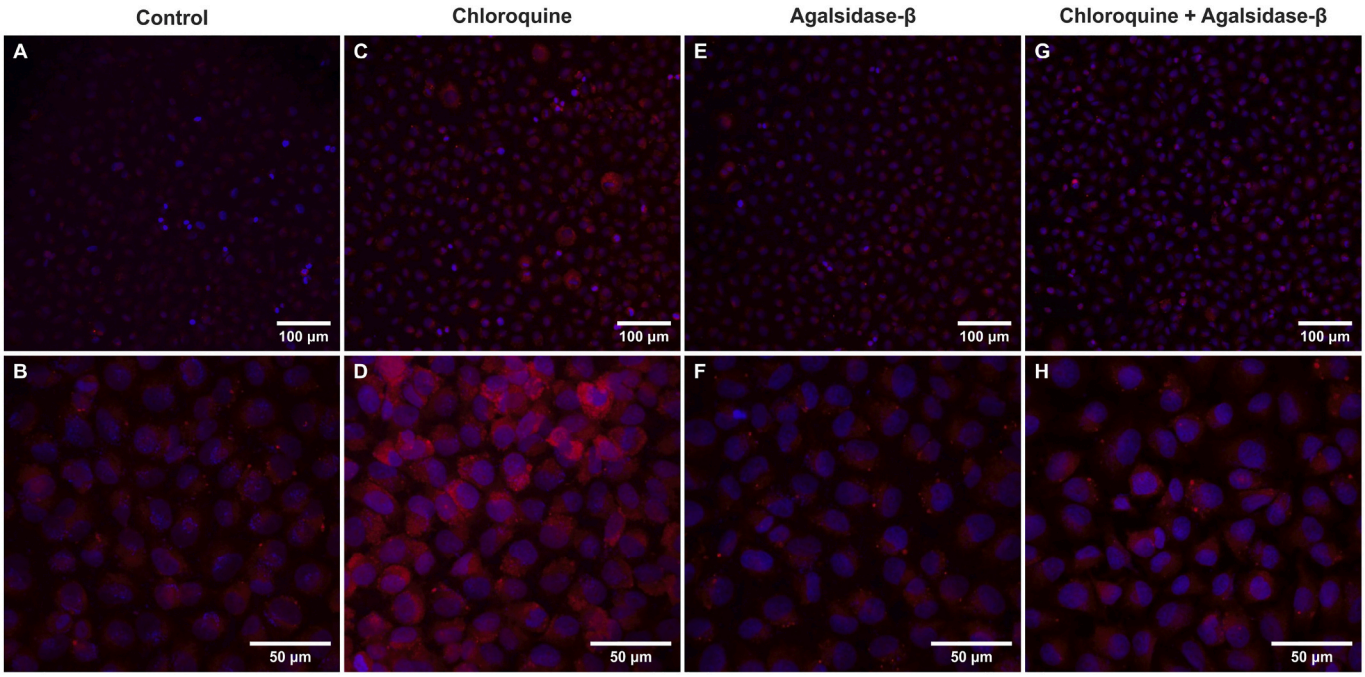

Fig. 3. Chloroquine promotes the accumulation of acid organelles in endothelial cells and this is prevented by agalsidase-β. Endothelial cells were exposed to chloroquine (0.5 μg/mL) and/or agalsidase-β (5 μg/mL). The control was considered as the untreated cells (A, B). Endothelial cells were treated with chloroquine (C, D), agalsidase-β (E, F), and with both compounds (G, H) for 72 h. Lysosomes (red) and nuclei (blue) were labelled with the Lysotracker probe and DAPI, respectively. Magnifications: A, C, E, G (200×, scale bar, 100 μm); B, D, F, H (600×, scale bar, 50 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

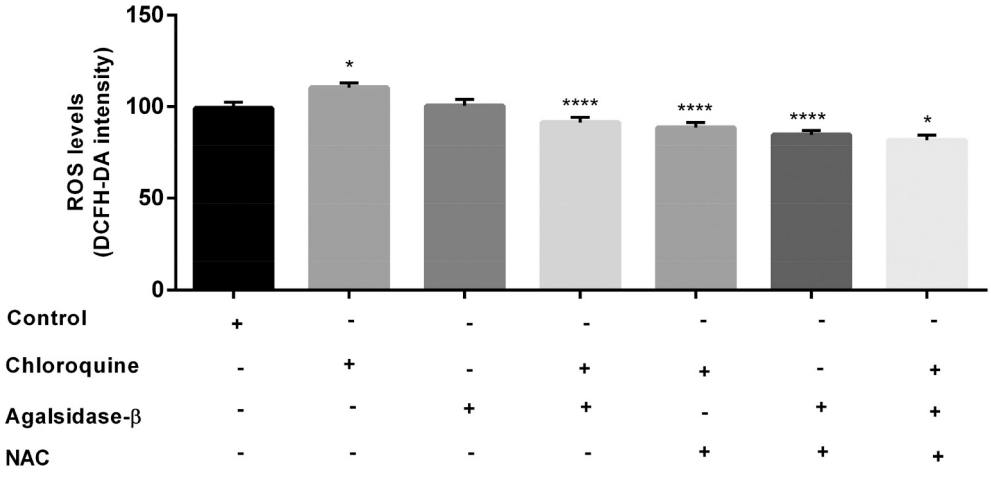

Fig. 4. Chloroquine increases ROS in endothelial cells and this is prevented by agalsidase-β. Endothelial cells were incubated with 1 μM DCFH-DA in PBS at 37 °C for 30 min and then treated with chloroquine (0.5 μg/mL) and/or agalsidase-β (5 μg/mL). ROS levels were determined immediately by measuring fluorescence (λEx 504 nm, λEm 524 nm). * $P < 0.05$, chloroquine *vs.* control; agalsidase-β, chloroquine with NAC *vs.* agalsidase-β and chloroquine; **** $P < 0.0001$, agalsidase-β and chloroquine *vs.* chloroquine; chloroquine with NAC (*N*-acetyl-L-cysteine, 2 mM) *vs.* chloroquine; agalsidase-β and NAC *vs.* chloroquine. ROS production in untreated control cells was assessed as 100%. Data are expressed as mean ± SEM of four independent experiments.

3.3. Agalsidase-β reversed lysosome inclusion accumulation in cultured endothelial cells exposed to chloroquine

To visually qualitatively assess lysosomal inclusions after treatment with chloroquine and/or agalsidase-β, lysosomes were labelled using the LysoTracker probe. Endothelial cells treated with media alone were used as negative controls (Fig. 3A and B). As shown in Fig. 3C and D, larger fluorescent organelles were visualized in chloroquine-treated cells. Additionally, the size of fluorescent organelles was reduced in cells treated with agalsidase-β and chloroquine in comparison to cells treated with chloroquine alone (Fig. 3G and H). Finally, cells treated only with agalsidase-β (Fig. 3E and F) exhibited fluorescence similar to controls.

3.4. Chloroquine-induced oxidative stress in cultured endothelial cells

To investigate the chloroquine and agalsidase-β effect on oxidative stress in endothelial cells, we evaluated intracellular ROS levels using the DCFH-DA probe. Fig. 4 shows that chloroquine significantly ($P < 0.05$) induced ROS production compared to that of control untreated endothelial cells. Additionally, agalsidase-β significantly decreased ROS production ($P < 0.0001$) in endothelial cells treated with chloroquine as compared to chloroquine alone. As a further control, pretreatment with the antioxidant NAC reduced cellular ROS levels in cells exposed to chloroquine ($P < 0.0001$) and or chloroquine/agalsidase-β ($P < 0.05$).

3.5. Chloroquine decreases NO levels in cultured endothelial cells

Chloroquine decreased in NO production ($P < 0.01$) in endothelial

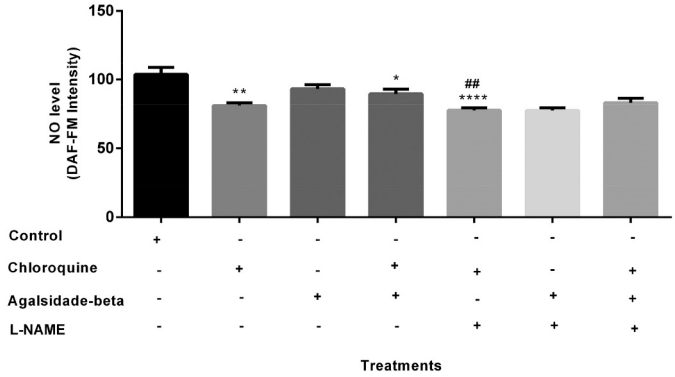

Fig. 5. Chloroquine decreases NO in endothelial cells and this is prevented by agalsidase-β. Endothelial cells were exposed to chloroquine (0.5 μg/mL) and/or agalsidase-β (5 μg/mL). NO levels were determined immediately by measuring fluorescence (λEx 495 nm, λEm 515 nm). ** $P < 0.01$, chloroquine *vs.* control; * $P < 0.05$, agalsidase-β and chloroquine *vs.* chloroquine; *** $P < 0.001$, chloroquine with L-NAME (100 μM) *vs.* chloroquine; $^{\#\#}$ $P < 0.01$, chloroquine with L-NAME (100 μM) *vs.* agalsidase-β and chloroquine. NO production in untreated control cells was considered to be 100%. Data are expressed as mean ± SEM of four independent experiments.

cells (Fig. 5), while co-treatment treated with agalsidase-β and chloroquine simultaneously significantly increased NO production compared to chloroquine alone ($P < 0.05$). As a control, L-NAME, an inhibitor of eNOS activity, also reduced NO levels ($P < 0.001$).

4. Discussion

The main findings of the present study are that chloroquine at *in vitro* test concentrations for COVID-19 causes lysosomal dysfunction and endothelial cell injury possibly through inhibition of the activity of the enzyme α-galactosidase (Fig. 6). Chloroquine induced the accumulation of acidic organelles, increased ROS levels, and decreased NO production, while agalsidase-β reverted these manifestations of endothelial cell stress.

Chloroquine was a short-lived promise for the treatment of COVID-19. Its low cost, widespread availability and perceived low toxicity made it part of most initial therapeutic regimens against COVID-19 as soon as non-controlled reports suggested its efficacy possibly due to its inhibitory effect on coronavirus replication *in vitro* (Colson et al., 2020). However, formal trials did not support its clinical efficacy (Rosenberg et al., 2020; Geleris et al., 2020). There are different potential explanations for the lack of efficacy in an *in vivo* setting, such as low *in vivo* drug exposure, limited by toxicity. We have now addressed a further hypothesis, that any potential benefit of chloroquine may be offset by an adverse impact on the endothelium. In this regard, the high mortality of severe COVID-19 has been related to micro- or macrothrombosis, *i.e.* to endothelial cell injury (Ackermann et al., 2020; Elsoukkary et al., 2020; Philipponnet et al., 2020). Although chloroquine was known to induce glycolipid deposits in lysosomes, the impact of this phenomenon on endothelial cell function had not been explored. The effect of chloroquine on decreasing NO production and increasing ROS levels are at least partially due to its inhibitory effect on the GLA gene encoding α-galactosidase A in endothelial cells (Shu et al., 2014), as demonstrated by the improvement of the chloroquine-induced endothelial cell dysfunction afforded by exogenous agalsidase. Importantly, chloroquine-induced endothelial cell stress was observed to be lethal at high concentrations. In this regard, this toxic effect might occur earlier in previously damaged cells, as is might be the case of infected endothelial cells or endothelial cells exposed to complement-mediated injury or hyperinflammation-induced injury in COVID-19 (Yu et al., 2020; Skendros et al., 2020).

Although the present study was focused on endothelial cells, it is likely that the findings can be extended to other cell types. Thus, given that chloroquine-induced endothelial cell stress was responsive to agalsidase, it is likely that α-galactosidase A hypoactivity is a contributor. As this enzyme is ubiquitously present throughout the body cells, it is then likely that target organs of COVID-19, such as the kidneys, the heart and the lungs may be adversely affected by chloroquine-induced cellular dysfunction in the course of the disease. Indeed, these cells were previously shown to accumulate glycolipids in individuals treated

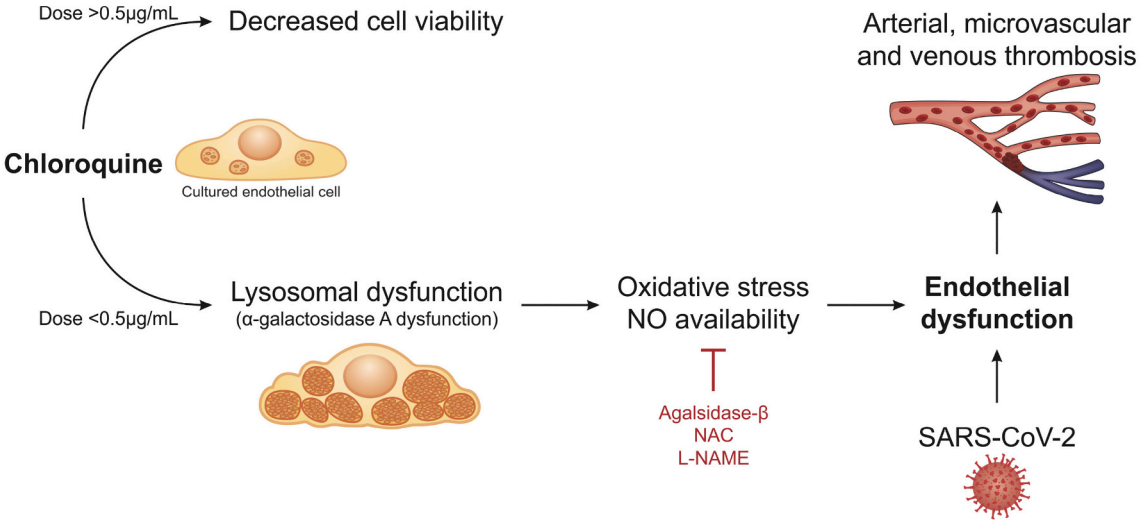

Fig. 6. Conceptual representation of study results. This study has shown that chloroquine is toxic to endothelial cells higher dose may induce endothelial cell death and it is likely that lower doses may be lethal for cells that have been previously damaged. A lower non-lethal but clinical relevant concentration promoted lysosomal dysfunction evidenced by increased lysosomal size, previously shown to correspond to glycolipid accumulation (Costa et al., 2013). This was associated with oxidative stress and decreased production of the vasodilatory molecule NO. A contributor to lysosomal dysfunction appears to be a hypoactivity of α-galactosidase A, as a recombinant for of the human enzyme, agalsidase, was protective. Overall, these changes may contribute to endothelial cell injury and dysfunction that has been identified as a key contributor to organ injury in COVID-19. Thus, while chloroquine may decrease viral replication one hand but promote a cytotoxicity that may be potential additive to that of viral infection. A working hypothesis is that this dual action of chloroquine (lysosomal toxicity *vs.* inhibition of viral replication) may impair the clinical benefit derived from the drug.

with chloroquine (Colson et al., 2020; Wang et al., 2020b). Thus, one may hypothesize that COVID-19-induced cell injury may lower the threshold for chloroquine-induced cytotoxicity which may in turn potentially aggravate the severity of COVID-19-induced tissue injury. Overall, our findings and hypotheses may explain at least in part the lack of benefits, as well as the potential harmful effect, of chloroquine in the clinical setting despite its antiviral activity *in vitro*.

We recognize that our study has some shortcomings. The expression and activity of α-GAL in the presence and absence of chloroquine and agalsidase-β were not evaluated. Moreover, it would had been interesting to use other cell viability assays such as Annexin V/PI and 7-AAD. To deeply assess oxidative stress, other methods could be used, such as lipid peroxidation and protein oxidation/nitration. Additionally, only one cell type was examined. However, as COVID-19 has been pointed as an endothelial disease (Lüscher, 2020) and endothelial cells are present in all target organs of COVID-19, reinforces the importance of using an endothelial model of chloroquine toxicity unlike other cell types that are organ-specific. Thrombosis and/or endothelial dysfunction are shared events of the different target organs of COVID-19 (Carriazo et al., 2020).

In conclusion, chloroquine may lead endothelial dysfunction due to oxidative stress and decreased NO production secondary to lysosomal accumulation of fatty acid substrates due to α-galactosidase A hypoactivity. Given the magnitude of endothelial cell injury in COVID-19, this adverse effect of chloroquine may contribute to its lack of clinical efficacy despite promising cell culture data.

Author contributions

P.C.G., G.B., R.S.C., B.B. and J.B. performed the formal analysis, investigation and methodology; P.C.G and R.S.C. performed the data curation and validation; P. C. G. performed the Writing - original draft; A.E.M.S., F.B., A.O. and M.D.S.N, performed Writing - review & editing. A.E.M.S. and F.B. were responsible by project conceptualization, administration, supervision and resources. All authors read and approved the content of the manuscript.

Funding

Paulo Cézar Gregório received PhD *sandwich* scholarships and Regiane Stafin da Cunha, Bruna Bosquetti received scholarships from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Júlia Budag received scholarship from Universidade Federal do Paraná/Tesouro Nacional.

Declaration of Competing Interest

AO is consultant for Sanofi Genzyme and Freeline and has received speaker fees from Amicus, MDSN has received speaker fees from Sanofi Genzyme. Other authors declare that they have no relevant financial interests.

References

Ackermann, M., Verleden, S.E., Kuehnel, M., Haverich, A., Welte, T., Laenger, F., et al., 2020. Pulmonary vascular endothelialitis, thrombosis, and angiogenesis in Covid-19. N. Engl. J. Med. 383, 120–128. https://doi.org/10.1056/NEJMoa2015432.

Biancini, G.B., Vanzin, C.S., Rodrigues, D.B., Deon, M., Ribas, G.S., Barschak, A.G., et al., 2012. Globotriaosylceramide is correlated with oxidative stress and inflammation in Fabry patients treated with enzyme replacement therapy. Biochim. Biophys. Acta Mol. basis Dis. 1822, 226–232. https://doi.org/10.1016/j.bbadis.2011.11.001.

Boulware, D.R., Pullen, M.F., Bangdiwala, A.S., Pastick, K.A., Lofgren, S.M., Okafor, E.C., et al., 2020. A randomized trial of hydroxychloroquine as postexposure prophylaxis for Covid-19. N. Engl. J. Med. 383, 517–525. https://doi.org/10.1056/NEJMoa2016638.

Carriazo, S., Kanbay, M., Ortiz, A., 2020. Kidney disease and electrolytes in COVID-19: more than meets the eye. Clin. Kidney J. 13, 274–280. https://doi.org/10.1093/ckj/sfaa112.

Chimenti, C., Scopelliti, F., Vulpis, E., Tafani, M., Villanova, L., Verardo, R., et al., 2015. Increased oxidative stress contributes to cardiomyocyte dysfunction and death in patients with Fabry disease cardiomyopathy. Hum. Pathol. 46, 1760–1768. https://doi.org/10.1016/j.humpath.2015.07.017.

Colson, P., Rolain, J.-M., Raoult, D., 2020. Chloroquine for the 2019 novel coronavirus SARS-CoV-2. Int. J. Antimicrob. Agents 55, 105923. https://doi.org/10.1016/j.ijantimicag.2020.105923.

WHO Coronavirus Disease, 2020. (COVID-19) Dashboard | WHO Coronavirus Disease (COVID-19) Dashboard. n.d. https://covid19.who.int/ (accessed October 15, 2020).

Costa, R.M., Martul, E.V., Reboredo, J.M., Cigarran, S., 2013. Curvilinear bodies in hydroxychloroquine-induced renal phospholipidosis resembling Fabry disease. Clin. Kidney J. 6, 533–536. https://doi.org/10.1093/ckj/sft089.

Desnick, R.J., 2015. Fabry disease. In: Rosenberg's Mol. Genet. Basis Neurol. Psychiatr. Dis. Elsevier, pp. 419–430. https://doi.org/10.1016/B978-0-12-410529-4.00038-3.

Elsoukkary, S.S., Mostyka, M., Dillard, A., Berman, D.R., Ma, L.X., Chadburn, A., et al., 2020. Autopsy findings in 32 patients with COVID-19: a single-institution experience. Pathobiology 1–13. https://doi.org/10.1159/000511325.

Fernandez-Fernandez, B., D'Marco, L., Górriz, J.L., Jacobs-Cachá, C., Kanbay, M., Luis-Lima, S., et al., 2020. Exploring sodium glucose co-Transporter-2 (SGLT2) inhibitors for organ protection in COVID-19. J. Clin. Med. 9, 2030. https://doi.org/10.3390/jcm9072030.

Geleris, J., Sun, Y., Platt, J., Zucker, J., Baldwin, M., Hripcsak, G., et al., 2020. Observational study of hydroxychloroquine in hospitalized patients with COVID-19. N. Engl. J. Med. 382, 2411–2418. https://doi.org/10.1056/NEJMoa2012410.

Germain, D.P., 2010. Fabry disease. Orphanet J. Rare Dis. 5, 30. https://doi.org/10.1186/1750-1172-5-30.

Horby, P., Mafham, M., Linsell, L., Bell, J.L., Staplin, N., Emberson, J.R., et al., 2021. Effect of Hydroxychloroquine in Hospitalized Patients with COVID-19: Preliminary Results from a Multi-Centre, Randomized, Controlled Trial. https://doi.org/10.1101/2020.07.15.20151852. MedRxiv 2020:2020.07.15.20151852.

Inagaki, M., Katsumoto, T., Nanba, E., Ohno, K., Suehiro, S., Takeshita, K., 1993. Lysosomal glycosphingolipid storage in chloroquine-induced α-galactosidase-deficient human endothelial cells with transformation by simian virus 40: in vitro model of Fabry disease. Acta Neuropathol. 85, 272–279. https://doi.org/10.1007/BF00227722.

Kang, J.J.-E., Shu, L., Park, J.L., Shayman, J.A., Bodary, P.F., 2014. Endothelial nitric oxide synthase uncoupling and microvascular dysfunction in the mesentery of mice deficient in α-galactosidase a. Am. J. Physiol. Gastrointest. Liver Physiol. 306, G140–G146. https://doi.org/10.1152/ajpgi.00185.2013.

Lüscher, T.F., 2020. Understanding COVID-19: in the end it is the endothelium—what else? Eur. Heart J. 41, 3023–3027. https://doi.org/10.1093/eurheartj/ehaa706.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63. https://doi.org/10.1016/0022-1759(83)90303-4.

Park, J.L., Whitesall, S.E., D'Alecy, L.G., Shu, L., Shayman, J.A., 2008. Vascular dysfunction in the α-GALACTOSIDASE a-knockout mouse is an endothelial cell-, plasma membrane-based defect. Clin. Exp. Pharmacol. Physiol. 35, 1156–1163. https://doi.org/10.1111/j.1440-1681.2008.04984.x.

Philipponnet, C., Aniort, J., Chabrot, P., Souweine, B., Heng, A.-E., 2020. Renal artery thrombosis induced by COVID-19. Clin. Kidney J. 13 https://doi.org/10.1093/ckj/sfaa141, 713–713.

Ravarotto, V., Simioni, F., Carraro, G., Bertoldi, G., Pagnin, E., Cal, L.A., 2018. Oxidative Stress and Cardiovascular-Renal Damage in Fabry Disease : Is There Room for a Pathophysiological Involvement?, pp. 1–7. https://doi.org/10.3390/jcm7110409.

Repetto, G., del Peso, A., Zurita, J.L., 2008. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nat. Protoc. 3, 1125–1131. https://doi.org/10.1038/nprot.2008.75.

Rodrigues-Diez, R.R., Tejera-Muñoz, A., Marquez-Exposito, L., Rayego-Mateos, S., Santos Sanchez, L., Marchant, V., et al., 2020. Statins: Could an old friend help in the fight against COVID-19? Br. J. Pharmacol. https://doi.org/10.1111/bph.15166 bph.15166.

Rosenberg, E.S., Dufort, E.M., Udo, T., Wilberschied, L.A., Kumar, J., Tesoriero, J., et al., 2020. Association of treatment with hydroxychloroquine or azithromycin with in-hospital mortality in patients with COVID-19 in New York state. JAMA 323, 2493–2502. https://doi.org/10.1001/jama.2020.8630.

Schieber, M., Chandel, N.S., 2014. ROS function in redox Signaling and Oxidative Stress. Curr. Biol. 24, R453–R462. https://doi.org/10.1016/j.cub.2014.03.034.

Shen, J.-S., Meng, X.-L., Moore, D.F., Quirk, J.M., Shayman, J.A., Schiffmann, R., et al., 2008. Globotriaosylceramide induces oxidative stress and up-regulates cell adhesion molecule expression in Fabry disease endothelial cells. Mol. Genet. Metab. 95, 163–168. https://doi.org/10.1016/j.ymgme.2008.06.016.

Shu, L., Vivekanandan-Giri, A., Pennathur, S., Smid, B.E., Aerts, J.M.F.G., Hollak, C.E.M., et al., 2014. Establishing 3-nitrotyrosine as a biomarker for the vasculopathy of Fabry disease. Kidney Int. 86, 58–66. https://doi.org/10.1038/ki.2013.520.

Skendros, P., Mitsios, A., Chrysanthopoulou, A., Mastellos, D.C., Metallidis, S., Rafailidis, P., et al., 2020. Complement and tissue factor-enriched neutrophil extracellular traps are key drivers in COVID-19 immunothrombosis. J. Clin. Invest. https://doi.org/10.1172/JCI141374.

Wang, Y., Zhang, D., Du, G., Du, R., Zhao, J., Jin, Y., et al., 2020a. Remdesivir in adults with severe COVID-19: a randomised, double-blind, placebo-controlled, multicentre trial. Lancet 395, 1569–1578. https://doi.org/10.1016/S0140-6736(20)31022-9.

Wang, M., Cao, R., Zhang, L., Yang, X., Liu, J., Xu, M., et al., 2020b. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. Cell Res. 30, 269–271. https://doi.org/10.1038/s41422-020-0282-0.

White, N.J., Watson, J.A., Hoglund, R.M., Chan, X.H.S., Cheah, P.Y., Tarning, J., 2020. COVID-19 prevention and treatment: a critical analysis of chloroquine and hydroxychloroquine clinical pharmacology. PLoS Med. 17, e1003252 https://doi.org/10.1371/journal.pmed.1003252.

Yu, J., Yuan, X., Chen, H., Chaturvedi, S., Braunstein, E.M., Brodsky, R.A., 2020. Direct activation of the alternative complement pathway by SARS-CoV-2 spike proteins is blocked by factor D inhibition. Blood. https://doi.org/10.1182/blood.2020008248.